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Women with germline mutations in *BRCA1* are strongly predisposed to cancers of the ovary and fallopian tubes. Given the strong link between menstrual activity and risk of ovarian cancer in the general population, we hypothesized that *BRCA1* might predispose to ovarian cancer indirectly, by influencing ovarian granulosa cells, which play an important role in controlling menstrual cycle progression. We used the *Cre-lox* system to inactivate the mouse *Brca1* gene in granulosa cells. A truncated form of the FSH receptor promoter was used as Cre driver. Our most recent results show that a majority (40 of 59) of mutant mice develop grossly visible cystic tumors either attached to the ovary or the uterine horns. These tumors resembled human serous cystadenomas, which are benign tumors made up of the same cell type as ovarian serous carcinomas. We confirmed that these tumors carried only the wild type allele of the floxed Brca1 allele while the mutant form was present in granulosa cells. These findings strongly support our initial hypothesis that Brca1 influences tumor development cell non-autonomously, through an effector secreted by granulosa cells. We developed tools such as long-term cultures of human granulosa cells, which will be used to compare the gene expression patterns of wild type and mutant granulosa cells in the second year. We also obtained preliminary data suggesting that the dynamics of the hormonal changes associated with the estrous cycle are slightly different in mutant mice, suggesting that the influence of granulosa cells on tumor predisposition in this animal model may be mediated through their role in the ovulatory cycle. Finally, we show evidence that the mutant mice show increased proliferative activity in epithelial cells lining the uterus and endometrium and endometrial glands, strongly supporting our view that ovarian epithelial tumors are derived from components of the mullerian tract.

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Introduction

Most individuals with familial predisposition to ovarian carcinoma carry a germline mutation in the *BRCA1* gene. In spite of extensive efforts to understand the normal function of the BRCA1 gene product during the last decade, the reason for the association between *BRCA1* mutations and disease predisposition are still unclear. In particular, there is no good explanation for the site specificity of the cancers that develop in these individuals. Indeed, although the *BRCA1* gene is expressed in most cell types, mutation carriers develop primarily breast and ovarian/fallopian tube tumors. We hypothesized that BRCA1 controls ovarian cancer predisposition in a cell non-autonomous manner, through a factor secreted by ovarian granulosa cells. The idea is that reduction in BRCA1 activity in granulosa cells results in changes in the secretion, by those cells, of one or several circulating or paracrine factors that influence the cell of origin of ovarian tumors. We tested this hypothesis by inactivating the *Brca1* gene in mouse ovarian granulosa cells specifically. We had reported, in our initial grant application, that over 50% of the mice carrying this targeted gene knockout developed ovarian/tubal tumors morphologically very similar to human ovarian serous cystadenomas in strong support of our hypothesis. We proposed to elucidate the mechanism of tumor predisposition in this mouse model by identifying the signaling molecules downstream of Brca1 that control tumorigenesis (aim #1) and to test the hypothesis that tumor development in this animal model results from an effect of Brca1 on the epithelial cells lining the entire mullerian tract. This latter hypothesis has important implications on the understanding of the exact site of origin of human ovarian epithelial tumors.

Body

The progress related to each task mentioned in the original statement of work is summarized below:

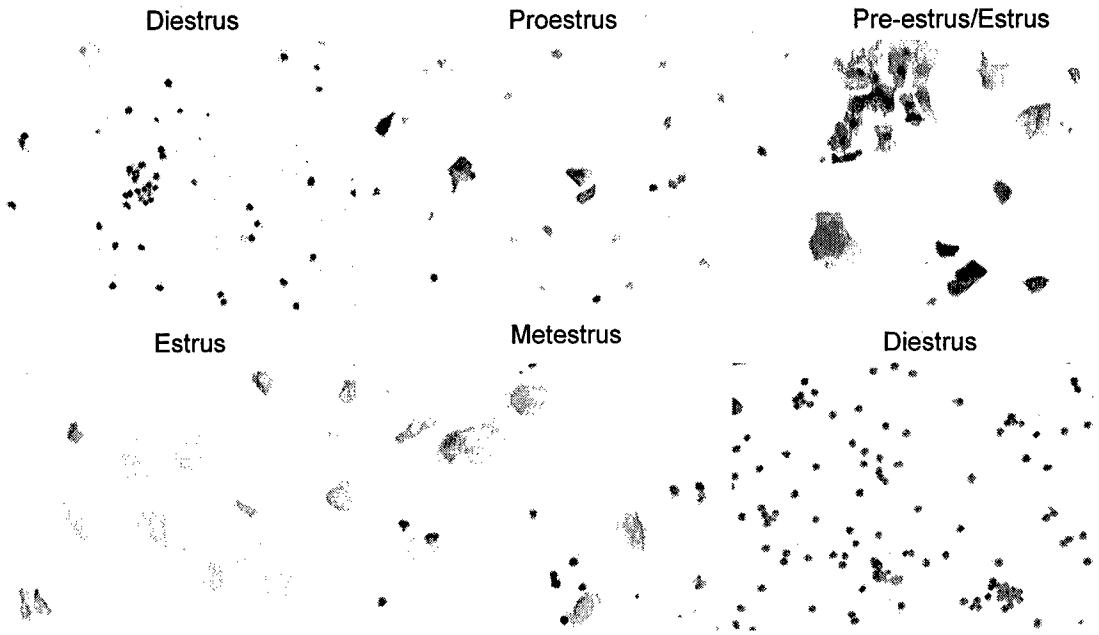
Task 1: Breed and genotype mice to support aims #1 and #2. According to our initial statement, this task was essentially meant to maintain our mouse colonies in order to ensure enough mice to support the other tasks. We expanded this task during the first year to also provide enough mice to confirm our initial experiments and further characterize the tumors that develop in the mutant mice. This was necessary to ensure our ability to publish this data in a respected peer reviewed journal. We had only examined 30 mice when we first submitted our proposal and although we had argued that the tumors that we had observed in the mutant animals were epithelial and not of granulosa cell origin, this had not been proven. We therefore maintained a colony of about 1000 mice (mutants and controls). We have doubled the number of mutant mice examined (from 30 to 59). In addition, although the mice that we had examined in the initial proposal were about 12 months of age, most of the mice examined during the first year of funding were between 15 to 22 months in order to test the hypothesis that tumor incidence would be higher in this more advanced age group. In addition, we have performed immunostains for various epithelial and granulosa cell markers on tumor samples to verify their cell lineage of origin and used PCR and laser capture microdissection technologies to confirm our suspicion that the mutant *Brca1* alleles were restricted to granulosa cells and, in particular, were absent in the ovarian and uterine tumors.

The results confirmed our earlier findings that the *Brca1* knockout in our mutant mouse population was restricted to granulosa cells. When genomic DNA from a total of 5 ovarian cysts and 3 uterine cysts was amplified enzymatically using primers specific for the mutant *Brca1* alleles, the only specific product seen was with one of the ovarian cysts. The presence of a product in this sample was probably due to the fact that this lesion had not been microdissected and was probably contaminated with ovarian stromal elements. None of the microdissected ovarian or uterine tumors examined showed evidence of *Brca1* rearrangement although all tumors showed a product when primers specific for the wild type allele were used. In contrast, products for the rearranged allele were readily obtainable with genomic DNA from ovarian stroma. The authenticity of these products were verified by DNA sequencing (see attached manuscript by Chodankar et al).

As of now, about 68% of the mutant mice developed either ovarian or uterine tumors or tumors in both of these organs. The tumors expressed nonsquamous keratins and did not express mullerian inhibiting substance (a marker of granulosa cells), attesting to their epithelial nature (see attached manuscript by Chodankar et al). In

addition, the tumors expressed estrogen and progesterone receptors. None of this data was available at the time of our first submission. We submitted this work to Current Biology (one of the Cell Press journals). Although the manuscript is not officially accepted yet, it was reviewed by this journal and the editors invited us to resubmit a revised manuscript after addressing reviewers' comments. This was done and the revised version is still under review.

Task #2: Test specific candidate hormones for their potential regulation by BRCA1 in vivo. The original plan was to measure circulating levels of various hormones at specific stages of the estrous cycle, which we proposed to evaluate based on the color of the vaginal mucosa. It turned out that this method of determining the stage of the estrous cycle is very inaccurate and subjective. We therefore learned to perform Papanicoulou stains (PAP stains) on vaginal lavages of mice and trained ourselves on interpreting these stains in order to more accurately evaluate the stage of the cycle. This method turned out to be very reliable and reproducible. We plan to report it in the "Method" section of a future manuscript comparing the differences in circulating hormonal levels between normal and mutant mice and anticipate that this technique will attract the interest of several scientists. An illustration of the cytopathological changes associated with each stage of the estrous cycle is illustrated in the following figure:

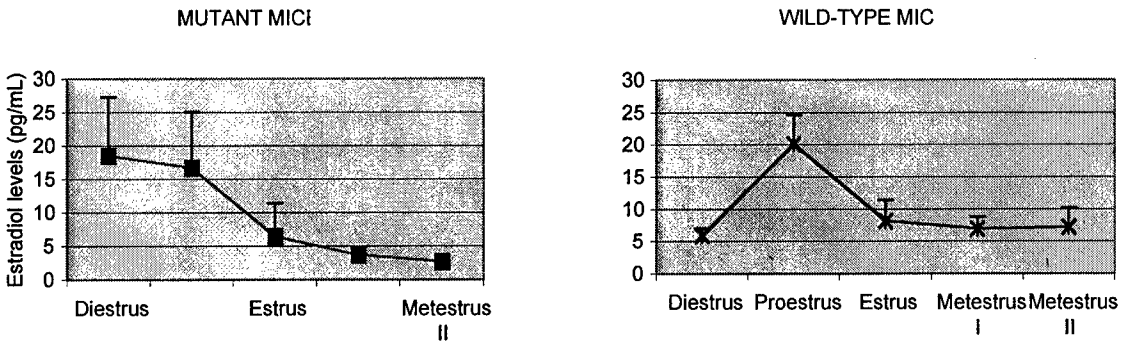


The diestrus and proestrus phases correspond to the follicular phase of the menstrual cycle in humans. Estrus corresponds to ovulation while metestrus corresponds to the luteal phase. Vaginal smears obtained at the diestrus phase show primarily inflammatory cells. Immature (green) epithelial cells start appearing at proestrus. By pre-estrus, the inflammatory cells have completely disappeared and an admixture of immature (green) and mature (orange) epithelial cells are seen. Estrus is characterized exclusively by mature epithelial cells. Metestrus, which is often subdivided into metestrus I and metestrus II, shows mature epithelial cells admixed with an increasing number of inflammatory cells. Although these changes have been described in the past using other staining techniques such as Giemsa, we are not aware of any previous report based on PAP stain, which greatly facilitates evaluation of parameters such as cellular maturity.

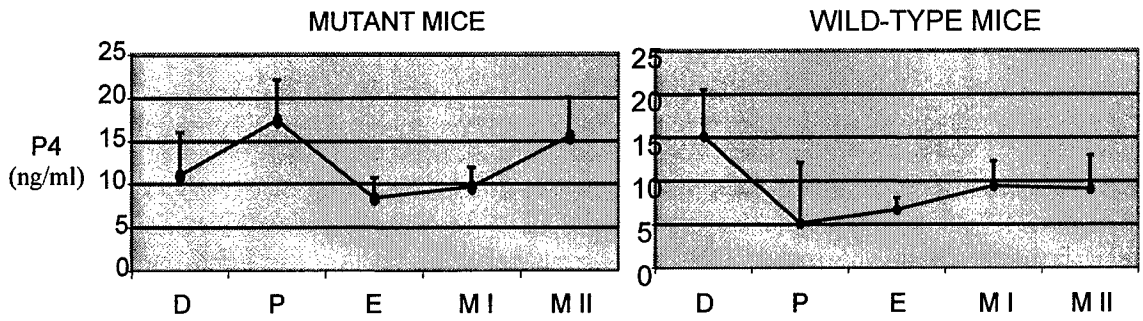
There has been some delay in completing the work initially proposed in this task because of the time it took to realize that our initial approach was problematic and to develop and train ourselves with the above method based on PAP smears. We nevertheless have not only initiated these proposed studies, but also expanded them in order to maximize the information. Our initial proposal was to examine and compare hormone levels in normal and mutant mice on a single day of the estrous cycle only. This was in part because our proposed method of evaluating the stage of the cycle only allowed identification of the estrous stage and was therefore

unreliable for the remaining stages of the cycle. After having familiarized ourselves with the alternative method described above that allows for accurate staging throughout the cycle, we realized that our studies would be much more informative if we looked at every stage of this cycle. The reason is that whereas our initially proposed studies allowed for measurement of absolute hormonal levels at a fixed time point, our modified approach can allow us to compare the timing and magnitude of hormonal fluctuations at all stages. We felt that this was important because of the possibility that the most important changes between normal and mutant mice would be in the dynamics of hormonal fluctuations as opposed to in absolute levels at a fixed time point.

The following illustration compares the levels of estradiol in mutant *versus* normal mice at each stage of the cycle



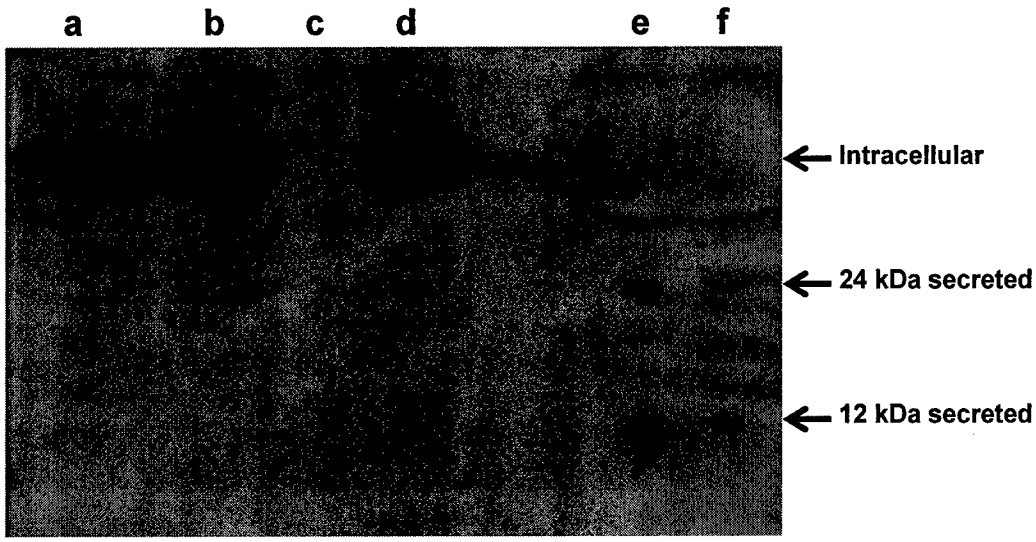
These results were obtained with 5 mutant and five wild-type mice. Although they are not statistically significant due to the small sample size, they do suggest that the peak circulating levels of estradiol occur earlier in mutant mice. We anticipate that we will be able to demonstrate statistical significance after examination of our initially proposed number of 25 mice in each group, which we expect to complete during the next year of the grant period. The blood samples were also examined for levels of progesterone. Again, the results suggested that circulating levels of this hormone peaked at different time points in mutant *versus* wild-type mice. The results are not statistically significant, but encourage us to complete this task using our proposed number of mice, which should allow us to reach statistical significance. Our current results for progesterone (P4) are shown in the following illustration:



We have so far focused our studies on estradiol and progesterone because of the difficulty of obtaining enough blood to allow measurement of all hormones mentioned in the initial proposal at the same time. These other hormones will be examined later after collecting additional blood samples from other age-matched mice.

Task #3: Test specific candidate hormones for their potential regulation by BRCA1 in vitro. The initial proposal was to remove both ovaries from one mutant mouse and one control mouse, harvest granulosa cells, and initiate several granulosa cell cultures per mouse in vitro. After ensuring purity of our cell cultures and verifying their authenticity, we would measure and compare the levels of hormones secreted in the conditioned medium in normal *versus* mutant mice. We have succeeded in obtaining cultures of granulosa cells as initially proposed. We were able to obtain primary cultures of such cells by using sterile needles to puncture ovarian follicles and aspirating the cell rich fluid with a syringe, followed by dispersing the cells and plating them in

tissue culture dishes. These cells can be cultured for at least 5 passages before undergoing senescence (we have not yet determined the upper limit of their *in vitro* longevity). The following illustration is a western blot of cells and conditioned medium recovered from two cultures (one from wild type and one from mutant mice) and probed with an antibody against mullerian inhibiting substance (MIS), which we use as marker of granulosa cells:



a: extract from cultured wild type granulosa cells
b: extract from cultured mutant granulosa cells
c: buffer only
d: extract from normal ovary
e: conditioned medium from cultured wild type granulosa cells
f: conditioned medium from cultured mutant granulosa cells

Lanes a and b, representing results with cell extracts, show expression of the 64 kd intracellular MIS. Lanes e and f were loaded with conditioned medium and show 12 kd and 24 kd fragments characteristic of the secreted forms of the protein, which are cleaved products of the intracellular form [1]. These results attest to the authenticity of the granulosa cell cultures obtained using our current protocol.

Although we attempted to compare the levels of various hormones released in conditioned medium for normal and mutant granulosa cell cultures, we realized that there is marked fluctuation in the amount of hormone released even when comparing cells from the same initial source. This may be due to differences in stages of follicular development of the granulosa cells from which the cultures were derived. Indeed, even if multiple cultures are derived from the same ovary, the various follicles used as source of granulosa cells to establish these cultures will undoubtedly be at different stages. The problem is magnified when comparing cultures for different mice because even if the mice are age-matched. Thus, if we had pursued our initially proposed approach, we would have had problems distinguishing effects of the mutant phenotype from normal phenotypic variability. We have therefore designed another approach to get around this problem. Our revised strategy is to obtain granulosa cell cultures for ovaries of mice homozygous for the floxed *Brca1* allele but lacking the *Cre* transgene. These mice express wild type Brca1 because of the absence of this transgene. The idea is to infect the cells with either a retroviral vector expressing *Cre* or with the empty vector. Since both vectors also express the green fluorescence protein, it is easy to separate the infected from uninfected cells in each group. We will compare cultures infected with these two vectors for hormonal synthesis and secretion. The advantage of this approach is that it will allow us to compare cells from the same original culture and differing only with regard to Brca1 function (only mice infected with the vector expressing *Cre* will have an inactive Brca1). We are currently in the process of making the appropriate retroviral vectors and testing this approach. Although this change in strategy has delayed progress with this task, we anticipate that once this is set up, results will be obtained not only more reliably but also more rapidly than they would have been with our initial approach, allowing us to catch up with our initial schedule. We expect to have characterized and tested our retroviral

reagents and to have strong data regarding the differences hormonal secretions between normal and mutant granulosa cells by the end of the second year of the grant period.

Task #4: Expression microarray analyses to compare gene expression in normal and mutant granulosa cells. This task entails performing expression microarray analyses of normal and mutant granulosa cells. The original plan was to use separate cultures of cells derived from normal and mutant mice. We realized that the problems encountered with task 3, which was initially based on using similar cell cultures, would probably apply to this task as well. We therefore modified our approach and plan to use the same culture system describe under task 3. We will be ale to initiate our microarray analyses as soon as the proposed retroviral vectors are characterized and tested, which should happen in the next few weeks.

Task #6: Comparing proliferation of specific mullerian-derived tissues and ovarian tissues in mutant versus normal mice. Our original plan was to compare the proliferation rate of various tissues of interest in the reproductive tract in normal and mutant mice by measuring phosphorylation of histone-3 (a marker of mitosis) using immunohistochemical techniques. We first compared various immunohistochemical approaches (various antibodies, immunofluorescence *versus* immunoperoxidase, etc) and also tested various alternative markers of cell cycle activity (cyclins, PCNA) during the first months of the grant period. We concluded that staining for PCNA using immunoperoxidase (as opposed to immunofluorescence) methods was the most reliable method. We initiated our proposed experiments by comparing the percentage of PCNA-positive cells in epithelial cells of the uterus at the diestrus *versus* estrus stages in age-matched mutant *versus* control mice at four months of age. The results are shown in the following Table:

	Wild Type		**Mutant**	
	Luminal cells	Glandular cells	Luminal cells	Glandular cells
Diestrus	41	37	84	81
Estrus	19	29	49	39

Each measurement in the above Table represent the percentage of positive cells showing diffuse nuclear staining for PCNA and is an average of 3 different measurements per mouse. The total number of cells counted in each measurement was 325. Two mutant mice and two wild type mice were examined in each group. Although the results are preliminary given the small number of mice examined, they are striking in that there seems to be a marked increase in PCNA expression in mutant mice, with over 80% of the mutant mice staining positively in diestrus compared to half this number in wild type mice. The number of PCNA-positive cells was smaller in estrus, in agreement with the results of Lai *et al.* [2], who showed that most PCNA staining in rat uterus was seen during the diestrus and proestrus phases. However, the number of positive cells in mutant mice at this stage was again twice the number seen in wild type mice. These results attest to our ability to obtain meaningful measurements of cell proliferative activity in various tissues at specific stages and support the idea that Brca1 inactivation in granulosa cells modifies the endocrine signals responsible for cell proliferation along the mullerian tract. These studies will be expanded to examine a larger number of mice of various ages during the next year of this grant.

Task 7: Comparing proliferation of uterine cysts in mutant mice ovariectomized at specific time points. We have recently ovariectomized 5 one-month old mice and 5 two-month old mice in order to initiate this task. We are behind schedule with this task because we concentrated our efforts on the other tasks mentioned above, some of which were expanded and others which required modifications in the approach. We expect to accumulate data in the context of this task during the second year of the grant period.

Key Research Accomplishments

- We overcame the embryonic lethality of Brca1 knock out mice by targeting such knock out specifically to granulosa cells.

- We showed that mice carrying a mutant *Brca1* in their granulosa cells developed tumors not in granulosa cells, but in epithelial cells lining the entire mullerian tract. We further showed that the tumors did not carry mutant *Brca1* alleles.
- Our preliminary results, which will be confirmed in the second year of this grant, show that the mutant mice show differences in the timing of hormonal changes associated with the estrous cycle and have increased proliferation of the epithelial cells lining their mullerian tract.
- We succeeded in culturing ovarian granulosa cells from normal and mutant mice and will take advantage of this tool to further elucidate the mechanism of tumor predisposition in mutant mice.
- We improved the techniques allowing accurate identification of the stage of the estrous cycle in mice by adapting staining techniques used for PAP tests in humans to these animals.

Reportable Outcomes

A manuscript describing our mouse model and arguing that Brca1 controls cancer predisposition in a cell non-autonomous manner was submitted to Current Biology. The manuscript was reviewed by this journal and the editors invited us to resubmit a revised manuscript after addressing reviewers' comments. This was done and the revised version is currently still under review.

An abstract based on this work was presented by Dr. Dubeau at the Mouse Model of Human Cancers Consortium – Gynecological Models Meeting in Puerto Rico in February 2004 This abstract was submitted following a formal invitation by the organizers of this meeting, who had heard about our work. A copy of the abstract is shown in the appendix.

Dr. Dubeau was invited as a guest grand round speaker to present the work done in the context of this grant at University of South Florida (January 2004) and at Fox Chase Cancer Center in December 2004. In addition, Dr. Dubeau was invited to speak about this work at the Lynne Cohen Foundation Symposium on the Emerging Role of Screening & Prevention in Women's Cancers held in New York in April 2004.

Conclusions

Our results confirm the preliminary data presented in our initial grant application and provide strong support, based on a novel experimental mouse model, for the idea that the reason why individuals with germline mutations in the *BRCA1* gene are predisposed to ovarian cancer is that the ensuing decrease in *BRCA1* gene dosage results in a disruption of normal cellular interactions between ovarian granulosa cells and the cells from which ovarian epithelial tumors originate. In other words, BRCA1 controls the secretion of one or several hormonal or paracrine factor(s) by granulosa cells that can influence ovarian tumor predisposition. Identification of this factor, which is the essence of our proposal, could lead to the development of a new test for the diagnosis of ovarian cancer predisposition or to novel approaches to prevent ovarian tumor development in *BRCA1* mutation carriers. We have made progress toward achieving this goal in the first year of this grant by developing reagents such as long term cultures of granulosa cells and others that are needed to complete our proposed studies and by obtaining preliminary evidence that sex steroid hormones are secreted differently in mutant mice. We also consolidated the data presented in the initial grant application.

Another important consequence of our results comes from the findings that mutant mice in our experimental model are predisposed to tumor development along their entire mullerian tract and from the demonstration that the presence of a *Brca1* mutation in ovarian granulosa cells results in increased proliferation in the uterine horns. These results strongly support the idea, initially proposed by Dr. Dubeau [3], that ovarian epithelial tumors originate in the mullerian tract as opposed to the ovarian surface. A better understanding of the exact cell type from which ovarian epithelial tumors originate should facilitate the identification of ovarian cancer precursor lesions and lead to better screening methods for their early detection. This, in turn, should have a profound impact on the morbidity and mortality associated with these cancers.

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Appendices

Manuscript:

Chodankar R, Kwang S, Sangiorgi F, Hong H, Yen H-Y, Deng C, Pike MC, Shuler CF, Maxson R, Dubeau L: Inactivation of Brca1 in mouse ovarian granulosa cells causes serous epithelial cystadenomas carrying functional Brca1alleles in the ovary and uterus (under second revision).

Abstract:

Chodankar R, Kwang S, Yen H-Y, Hong H, Deng C, Sangiorgi F,Yu MC, Maxson R, Dubeau L: Homozygous Knock Out of *Brca1* in mouse ovarian granulosa cells results in benign and malignant ovarian epithelial tumors. Presented at the Mouse Model of Human Cancers Consortium – Gynecological Models Meeting in Puerto Rico in February 2004.

Inactivation of Brca1 in mouse ovarian granulosa cells causes serous epithelial cystadenomas carrying functional Brca1 alleles in the ovary and uterus

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Running head: cell non-autonomous control of tumorigenesis by Brca1

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Summary / Introduction

Women with germline mutations in *BRCA1* have a 40% risk of developing ovarian cancer by age 70 [1] and are also predisposed to cancers of the fallopian tubes [2-4]. Given that ovulatory activity is a strong risk factor for sporadic ovarian cancer [5], we hypothesized that reduced BRCA1 expression might predispose to gynecological cancers indirectly, by influencing ovarian granulosa cells. These cells secrete sex steroids that control the ovulatory cycle and influence the growth of ovarian epithelial tumors [6, 7]. Granulosa cells also secrete mullerian inhibiting substance (MIS), a hormone that inhibits both the formation of female reproductive organs in male embryos [8] and the proliferation of ovarian epithelial tumor cells [9, 10]. We tested this hypothesis by using the *Cre-lox* system to inactivate the *Brca1* gene in mouse ovarian granulosa cells. A truncated form of the FSH receptor promoter [11] served as the Cre driver. Here we show that indeed, inactivation of the *Brca1* gene in granulosa cells led to the development of cystic tumors in the ovaries and uterine horns. These tumors carried normal *Brca1* alleles, supporting the view that Brca1 may influence tumor development indirectly, possibly through an effector secreted by granulosa cells.

Results

Granulosa cell specificity of truncated Fsh receptor promoter

We verified the cell-type specificity of a truncated form of the Fsh receptor promoter shown previously to direct expression exclusively in granulosa cells [11]. We crossed a transgenic mouse expressing the Cre recombinase under the control of this promoter fragment with the ROSA26R Cre reporter mouse strain [12]. Examination of the pelvic organs of mice carrying the Cre driver and reporter showed beta galactosidase activity exclusively in granulosa cells (figure 1).

Consequences of loss of Brca1 in granulosa cells on tumor formation

Fshr-Cre transgenic mice were crossed with mice carrying a floxed *Brca1* allele [13] to create a *Brca1* homozygous knockout restricted to granulosa cells. One ovary was removed from each of 30 *Brca1 flox/flox; Fshr-Cre* mice at 2 months of age. Histological examination revealed that these ovaries were morphologically normal (not shown). The mice were fertile and, at least during the first 6 months of life, produced litters of normal size.

Fifty nine *Brca1 flox/flox; Fshr-Cre* mice, including the 30 mice that had a unilateral oophorectomy at two months, were sacrificed between the ages of 12 and 20 months. Forty of these 59 (68%) homozygous mutant mice had grossly visible cysts either attached to the ovary, within the wall of the uterine horns, or on the external surface of the uterine horns (figure 2). The ovarian cysts were occasionally bilateral

(figure 2). The uterine cysts were usually multiple and most concentrated near the ovaries. All cysts were lined by cuboidal to columnar cells and were occasionally papillary (see figure 3e below). The cysts resembled human serous cystadenomas, which are benign tumors composed of the same cell type as ovarian serous carcinomas.

A solid tumor contiguous to a morphologically benign cyst was observed in a single case. Although it showed levels of complexity and cellular atypia compatible with a malignant process in its solid component (figure 2d), the malignant potential of this tumor remains unclear because it showed no evidence of either invasion of surrounding structures or metastasis. Renal cysts were also observed in two mutant mice. No abnormality was seen in any of 36 age-matched littermate controls lacking *Cre* recombinase.

Evidence for a cell non-autonomous mechanism of tumor induction

Our studies with the R26R reporter mouse (Fig. 1) suggested that rearrangement of the *Brca1* gene in response to Fshr-Cre occurred primarily in ovarian granulosa cells, our intended target. That all tumors exhibited an epithelial morphology suggested that they were not derived from granulosa cells. Further support for this possibility came from findings that the tumor cells (i) expressed keratins (figure 3), which are markers of epithelial cells, and (ii) did not express mullerian inhibiting substance, a marker of granulosa cells (figure 3). The tumor cells also expressed estrogen (figure 3) and progesterone (not shown) receptor proteins, further supporting the view that they were functionally similar to human ovarian epithelial tumors.

The conclusion that the tumors did not originate in granulosa cells was also supported by the fact that they were often localized in the uterine horns, which do not contain granulosa cells. The possibility remained that the Fshr-Cre transgene produced a level of Cre that was sufficient to cause recombination of the floxed Brca1 allele but was too low to cause recombination of the R26R allele. If this were the case, then the tumor cells should carry the recombined form of the floxed *Brca1* allele. However, although the expected 530 bp product from unrearranged *Brca1* allele could be amplified readily from all tissues examined using PCR primers specific for this allele, the only tissues from which the expected 640 bp product from the rearranged allele could be amplified were whole ovaries, the site of granulosa cells, as well as one of four ovarian cysts that had been separated from the adjacent ovaries with scissors under a dissecting microscope (figure 4, bottom panel). The weak amplification product obtained with primers specific for the rearranged allele (pair e-d) in this ovarian cyst most likely reflects the presence of admixed ovarian stroma, either in the cyst wall or in contaminating fragments of normal ovary. It is highly unlikely that this allele played a role in tumor development given its absence in most cystic tumors examined. A fifth ovarian cyst subjected to laser capture microdissection to ensure absence of admixed granulosa cells did not contain the rearranged allele either in the lining epithelium or in the cyst wall (figure 4, middle panel). We detected only the unrearraged allele in the epithelial lining of two additional uterine cysts examined after laser capture microdissection (not shown).

Discussion

Our results strongly support our hypothesis that inactivation of the *Brca1* gene in granulosa cells acts cell-non-autonomously by altering the activity of an effector that influences tumorigenesis in cells from which ovarian epithelial tumors originate. This conclusion is based on the fact that inactivation of *Brca1* in ovarian granulosa cells led to the formation of epithelial tumors carrying normal *Brca1* alleles. An earlier report showed similarly that breast tumors resulting from inactivation of Brca1 in a subset of mammary cells (with MMTV-Cre or Wap-Cre) did not carry the mutant form of *Brca1* [13]. Although we did not examine breast tissue in *Brca1 flox/flox; Fshr-cre* mice, we note that ovulatory activity, which is controlled largely by the activity of granulosa cells, is a well-established risk factor for breast cancer in humans.[14].

That mice lacking a functional Brca1 protein in their granulosa cells developed lesions involving the uterine horns in addition to the ovaries is consistent with the observation that pre-cancerous changes are frequently seen in the fallopian tubes of women who are asymptomatic carriers of *BRCA1* mutations [2-4]. This is also compatible with the suggestion made earlier by one of us that ovarian epithelial tumors do not originate from the mesothelial layer lining the ovarian surface, the site favored by most authors, but from mullerian duct derivatives surrounding the ovary or abutting to this organ [15].

Most tumors that develop in individuals with germline *BRCA1* mutations and that show loss of heterozygosity at the *BRCA1* locus have retained the mutant allele [16-18], suggesting that BRCA1 may act as a classical tumor suppressor. However, not all tumors that develop in this group of patients carry losses of heterozygosity at this locus [18] and there is little evidence for the notion that the wild type allele in these tumors is silenced by epigenetic mechanisms [19]. In addition, a number of observations suggest that total loss of BRCA1 activity does not promote, but interferes with cellular growth. The small number of breast or ovarian cancer cell lines lacking a functional *BRCA1* gene that have so far been isolated typically have long doubling times. Primary cultures derived from *Brca1*$^{-/-}$ mouse embryos do not proliferate unless the embryos also carry a p53 knockout. Given that cells from such embryos grow only clonally, additional events must occur to ensure their viability [20, 21]. Recent evidence suggests that down-regulation of BRCA1 results in growth arrest at the G2 to M transition [22], a finding clearly inconsistent with the view that Brca1 functions as a classical tumor suppressor. That mutations in this ubiquitously expressed gene lead mainly to predisposition to breast and ovarian cancer is also difficult to reconcile with this view.

It is possible that the Fshr promoter used in our studies is expressed in cells other than granulosa cells at levels undetectable with the R26R reporter mouse. Thus non-granulosa cells may control ovarian and uterine tumorigenesis. However, we favor the hypothesis that it is the granulosa cells that act at a distance to control mullerian epithelial tumorigenesis via a mechanism regulated by Brca1. At least in reproductive organs, these cells appear to be the principal site of Brca1 inactivation. The idea that a specific effector

released by granulosa cells and regulated by Brca1 influences tumor predisposition in the mullerian tract is both the simplest and biologically most attractive hypothesis that follows from our data. Another possibility is that an abnormal Brca1 expression might result in changes in the dynamics of the estrus/menstrual cycle such as, for example, changes in the length of a specific phase, which in turn might influence tumor predisposition.

The finding that loss of Brca1 in mouse ovarian granulosa cells causes epithelial tumors in wild type cells of the ovary and uterus raises the prospects that reduced levels of functional BRCA1 protein in humans carrying a germline *BRCA1* mutation could lead to the development of cancer by modulating the ability of granulosa cells to act on distant target tissues. This hypothesis has important implications for the clinical management of individuals with a familial predisposition to ovarian tumors due to germline *BRCA1* mutations. Knowing the identity of the endocrine or paracrine factor(s) that mediate such actions-at-a-distance could provide a new way to identify individuals predisposed to ovarian cancer and could also form the basis for novel strategies, based on manipulations of the levels of the factor(s) in question, aimed at preventing ovarian cancer in individuals with familial predisposition to this disease.

Experimental Procedures

Immunohistochemical analyses.

The mouse monoclonal antibody against non-squamous keratins was purchased from Chemicon International (Temicula, CA, MAB 1611). The polyclonal goat antibody against mouse mullerian inhibiting substance was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, cat# sc-6886). Goat polyclonal antibodies against mouse estrogen receptor alpha and progesterone receptor proteins were purchased from Santa Cruz Biotechnology (cat# sc-542 and sc-2018 respectively. All primary antibodies were diluted 1:200. For secondary antibodies, we used either anti-mouse IgG purchased from Chemicon International (cat# AP124B) diluted 1:500 or anti-goat IgG purchased from Santa Cruz Biotechnology and diluted 1:200. Antibody binding was detected using the ABC kit (Vector Laboratories, Burlingame, CA).

Examination of *Brca1* rearrangement status by PCR

Tissues of interest were either microdissected using a Pixcell II laser capture microdissection instrument (Arcturus Bioscience, Inc., Mountain View, CA) or were sampled under a dissecting microscope. All laser capture microdissections were performed on tissues fixed in ethanol only, embedded in paraffin, and either unstained or lightly stained with hematoxylin. Genomic DNA was amplified by PCR using primers specific for either the unrearranged (primers a-b) or rearranged (primers e-d) alleles. The sequences of primers a and b were as published [13]. The sequence of primer e (forward)

was: 5'-GCAGTGAAGAGAACTTGTTCCT-3'. The sequence of primer d (reverse) was: 5'-CTGCGAGCAGTCTTCAGAAAG-3'. PCR cycling profiles were 30 seconds at 94°C, 60 seconds at 58°C, and 60 seconds at 72°C over 35 cycles.

Generation of mice with *Brca1* knock out targeted to granulosa cells

A transgene composed of the Cre recombinase protein-coding sequence (1.1 kb) and a 900 bp SV40 fragment containing an untranslated exon sequence and polyadenylation signal fused with a truncated form [11] of the FSH receptor promoter (285 bp) was used to produce transgenic mice. The initial parental mice were from a cross between C57 and Black 6 strains. Six lines were initially created, two of which were crossed with R26R reporter mice and found to be equally effective at driving *Cre*. One line was selected randomly for breeding with a mouse carrying a floxed *Brca1* allele described earlier [21]. The mouse genotypes were determined by amplifying tail DNA with primers specific for either the floxed *Brca1* allele or for *Cre*.

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Figure Legends

Figure 1: Specificity of Fsh receptor promoter for granulosa cells. Two transgenic mouse lines expressing Cre recombinase under the control of a truncated form [11] of the Fsh receptor promoter (285 bp) were crossed with a ROSA26R Cre reporter mouse. The pelvic organs were removed at 8 weeks postnatal and examined for the presence of *lacZ* under bright field (A, B, D) or dark field (E-F). Shown here are representative results from one transgenic line. A: ovaries with portion of adjacent uterine horns, showing *lacZ* staining restricted to the ovaries (arrows); B: whole mount section of one ovary showing scattered foci of *lacZ*; C: histological section of a mouse ovary stained with an antibody against mullerian inhibiting substance, a marker of granulosa cells; this panel is meant to illustrate the normal histology of ovarian follicles for use as reference when examining panels D to F; it shows 2 ovarian follicles (short arrows) each with a central cavity (long arrows) surrounded by immunopositive granulosa cells; cells outside the two ovarian follicles are ovarian stromal cells; D: serial sections through an entire ovarian follicle morphologically similar to those shown in C and showing *lacZ* staining confined to the granulosa cells; E: whole mount section through an entire ovary seen under dark field microscopy showing the presence of *lacZ* in ovarian follicles; the area within the rectangle, which shows a cross section through the center of one follicle as well as small portions of adjacent follicles, is enlarged in F.

Figure 2: Examples of ovarian and uterine lesions observed in mutant mice. Shown are gross photographs of ovarian (arrows in a-b) and uterine (arrows in e) cysts and

histological sections from ovarian (c-d) and uterine (f-h) lesions. The ovarian tumor shown in (a) was 80% cystic and 20% solid. Histological sections of both of these components are shown in c and d respectively. A bilocular cyst on the external surface of a uterine horn is shown under low and high magnification in panels f and h respectively. A uterine horn containing multiple epithelial cysts (arrows) is shown at low magnification in g; ec: endometrial cavity. Bars in panels a, b, and e are 5 mm. Bars in panels c, d, and h are 40 microns. Bars in panels f and g are 1000 microns. Stain: hematoxylin and eosin.

Figure 3: Immunohistochemical characterization of ovarian tumors. Panels a-e are various portions of the ovarian tumor shown in Fig. 2a, including a papillary area of the cystic component (e). The sections were stained with a polyclonal antibody against non-squamous keratins (a, c), and with monoclonal antibodies against either mullerian inhibiting substance (b, d), or against the estrogen receptor protein (e). The cytoplasmic staining pattern seen in e is similar to that seen in sections of normal endometrium from wild type mice (f). Secondary ovarian follicles of a mutant mouse stained with an antibody against mullerian inhibiting substance were shown in figure 1c. Bars are 40 microns in all panels except in e, where it is 100 microns.

Figure 4: Recombination status of floxed Brca1 alleles in normal and neoplastic tissues. The top diagram shows *lox*P sites flanking exon 11 of the *Brca1* gene. The arrowheads represent the position and orientation of oligonucleotide primers relative to the *lox*P sites. Genomic DNA from various normal or neoplastic tissues was amplified by

PCR using either primer pair e-d, specific for the rearranged *Brca1* allele, or pair a-b, specific for the unrearranged allele. The PCR products were resolved on agarose gels and visualized under UV after staining with ethidium bromide (middle and bottom panels). Lanes labeled "Ov cyst lining" and "endometrium" in the middle panel represent respectively the epithelial lining of an ovarian cyst and of endometrium separated from adjacent tissues by laser capture microdissection. The stromal cells underlying the ovarian cyst epithelium were likewise microdissected and examined ("Ov cyst wall"). The bottom panel shows four ovarian cysts not subjected to laser capture microdissection, but separated from the adjacent ovaries with scissors under a dissecting microscope. Although the expected 530 bp unrearranged *Brca1* allele (primer pair a-b) was detected in all tissues examined, the expected 647 bp amplification product from the rearranged allele (primer pair e-d) was only seen in normal ovarian tissues (which contains granulosa cells) as well as in one of four ovarian cysts (bottom panel). The product of approximately 300 bp in "Ov cyst lining" (primer pair e-d, middle panel) was sequenced, confirming that it represents a non-specific product. The 647 bp product from normal ovaries using the same primers was also sequenced and shown to be authentic.

Figure 1:

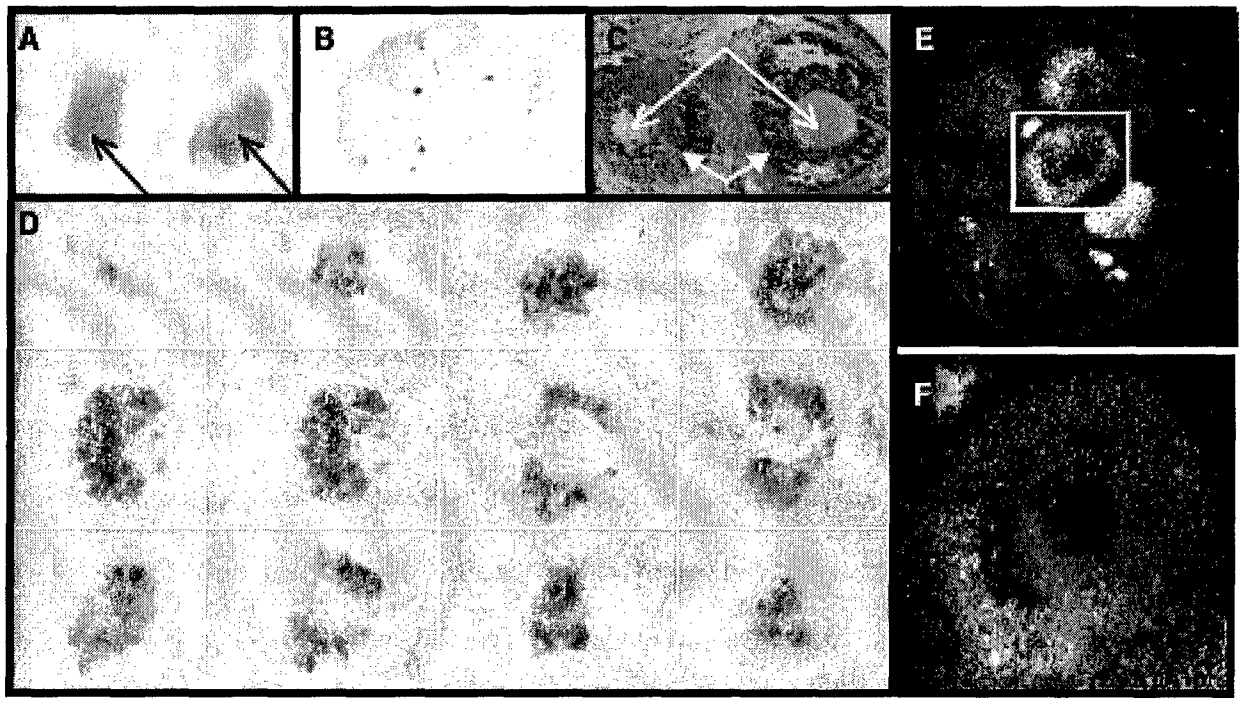


Figure 2:

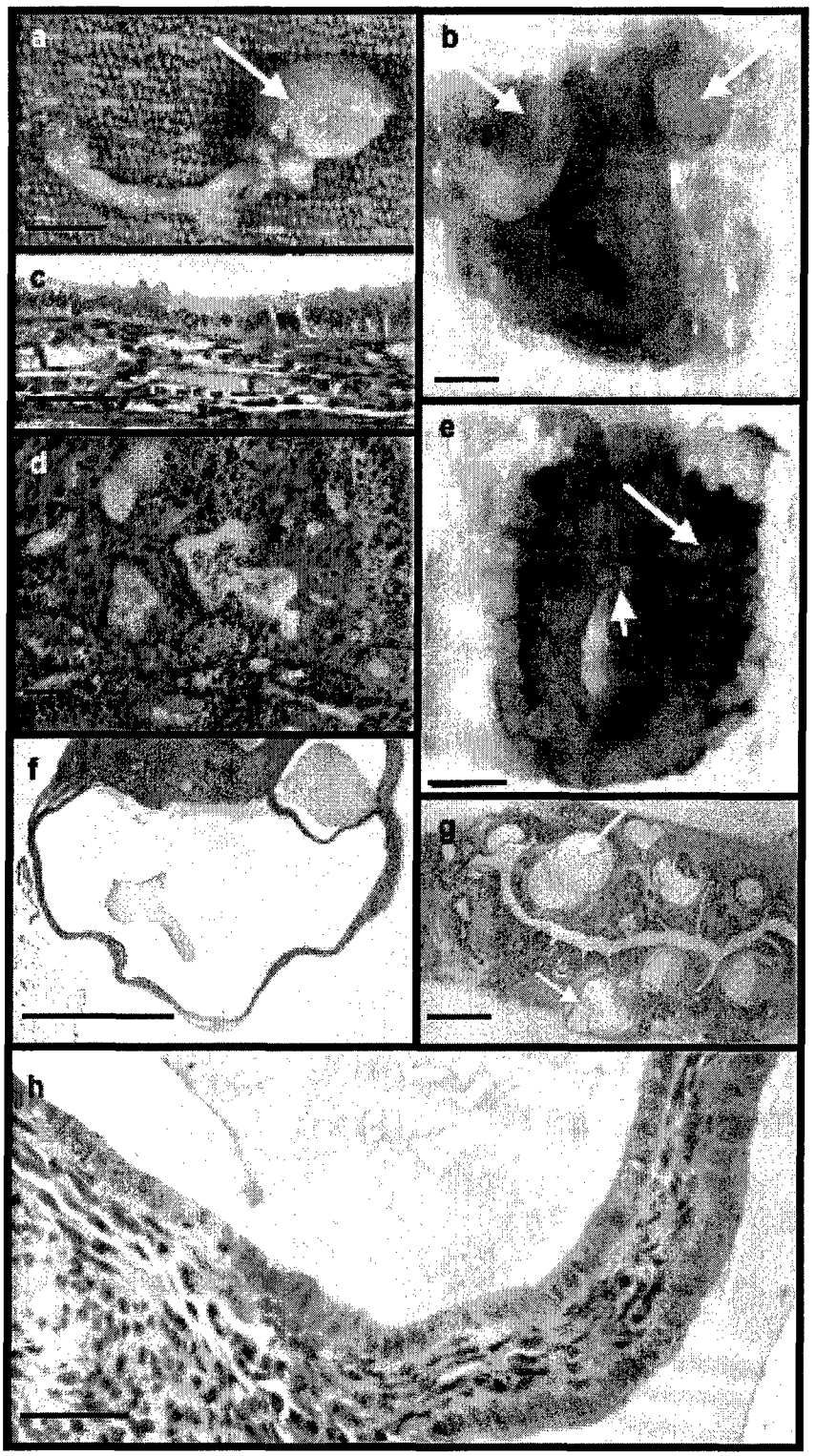


Figure 3

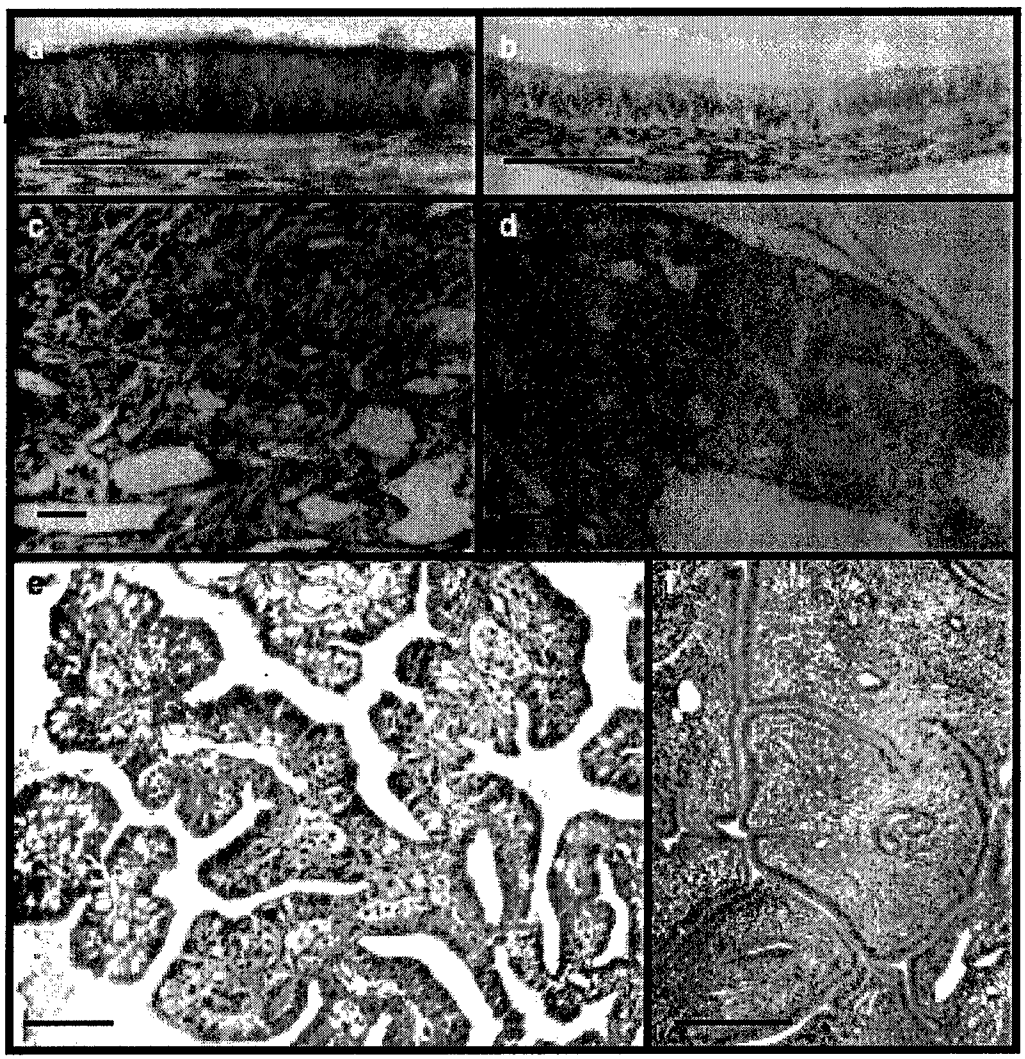
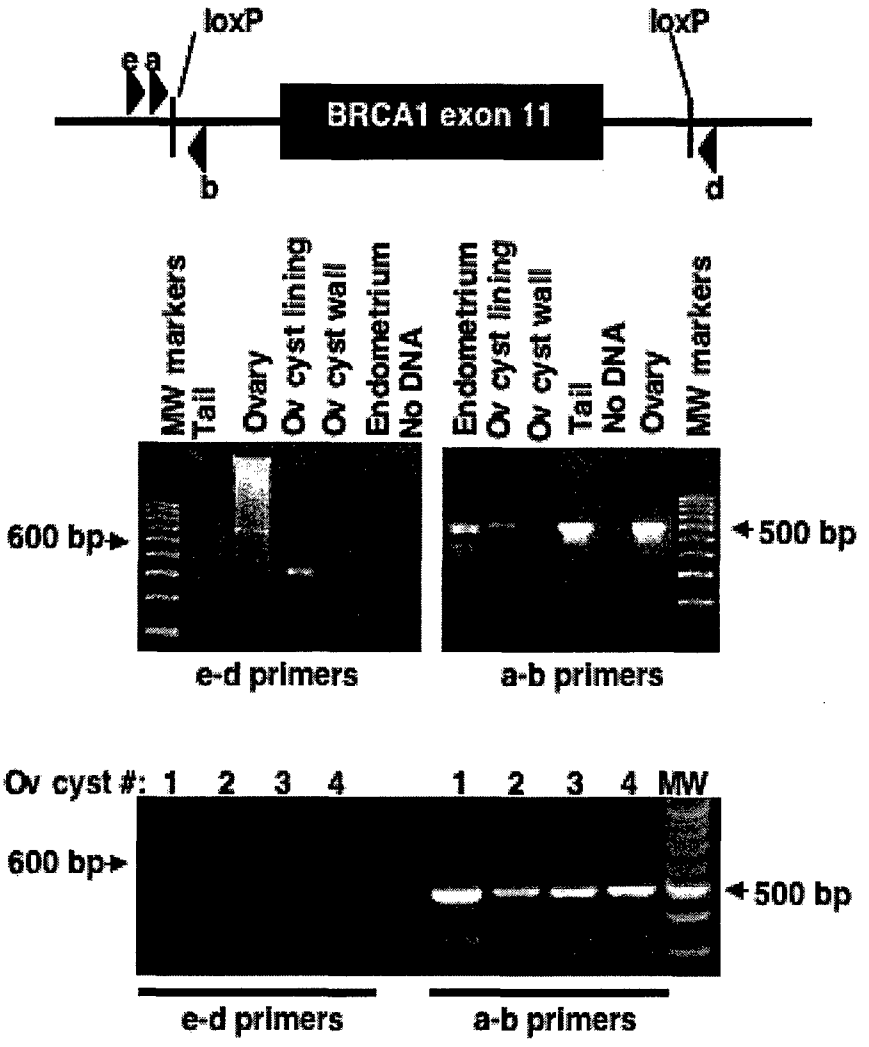


Figure 4: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